Interplay of Insulin-Like Growth Factor-II, Insulin-Like Growth Factor-I, Insulin-Like Growth Factor-I Receptor, COX-2, and Matrix Metalloproteinase-7, Play Key Roles in the Early Stage of Colorectal Carcinogenesis

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ABSTRACT

Purpose: The aim of this study was to characterize the relationship of insulin-like growth factor (IGF)-II expression with IGF-I, IGF-I receptor (IGF-IR), cyclooxygenase-2 (COX-2), and matrix metalloproteinase (MMP)-7 in early colorectal carcinogenesis.

Experimental Design: With the semiquantitative reverse transcriptase-PCR, 90 human colorectal tumor tissues (63 adenomas and 27 submucosal pT1 cancers) were analyzed for IGF-II, IGF-IR, IGF-I, COX-2, and MMP-7 expression. Ninety-nine adenoma tissues and 60 pT1 cancer tissues were also analyzed immunohistochemically for IGF-II expression. Paired carcinoma and adenoma tissues obtained from a carcinoma in adenoma lesion was analyzed by a cDNA array.

Results: IGF-II mRNA expression was detected in 37.8% of the 90 colorectal tumor tissues. The frequency of IGF-II mRNA expression was significantly higher in pT1 cancer (70.4%) than in adenoma (23.8%). Immunohistochemical IGF-II expression was also more frequently detected in pT1 cancer (58.3%) than in adenoma (25.3%). Loss of imprinting of the IGF-II gene was observed in 15 (44.1%) of 34 colorectal tumors in which IGF-II was overexpressed and the maternal one silent (10). Loss of imprinting, an epigenetic alteration, has been suggested to be the main mechanism underlying IGF-II overexpression. It has been reported that 44% of colorectal cancer patients showed loss of imprinting of IGF-II (10).

IGF-II exerts its mitogenic activity through the IGF-IR (11, 12). IGF-IR is a member of the tyrosine kinase receptor family (13) and is overexpressed in colon cancer mucosa compared with its expression level in normal or adenomatous mucosa (4, 14). Blockade of the IGF-II/IGF-IR axis by soluble IGF-IR reportedly inhibits growth of colon cancer xenografts in vivo (15). These results indicate that IGF-II/IGF-IR axis plays crucial roles in the growth and invasion of cancer cells (16–18).

Among mechanisms that regulate IGF-IR expression, the phosphatidylinositol 3'-kinase (PI3k)/Akt pathway plays a crucial role in its expression (19). COX-2 activates the PI3k/Akt pathway through prostaglandin E2 (PGE2) in colon cancer cells (20), indicating that COX-2/PGE2 is involved in IGF-IR expression. Mounting evidence indicates that COX-2 plays an important role in colorectal carcinogenesis (21, 22). COX-2 is overexpressed in 80 to 90% of colorectal cancers and in 40 to 50% of premalignant adenomas (21). Inactivation of the COX-2 gene expression was detected in 42.2% and 77.8% of the tumor tissues, respectively, and both were positively correlated with IGF-I, IGF-II, and IGF-IR expression. IGF-II was the most differentially expressed gene between carcinoma and adenoma lesions.

Conclusions: IGF-II, in conjunction with IGF-IR, IGF-I, COX-2, and MMP-7, seems to play a key role in the early stage of colorectal carcinogenesis.
in mice is associated with decreased intestinal tumorigenesis (23). Reduced prostaglandin biosynthesis through inhibition of COX-2 activity is thought to be the molecular basis for the chemopreventive effects of nonsteroidal anti-inflammatory drugs (NSAID) on colorectal carcinogenesis in both humans and rodents (21, 22). Moreover, NSAIDs reportedly reduce IGF-IR expression in vitro and also inhibit IGF-II–stimulated growth and invasion in a dose-dependent manner (24).

Alterations in each level of the IGF axis have been implicated in cancer development and progression. IGF binding proteins (IGFBP) have affinities for IGFs that are either equal to or stronger than those of the IGF receptors, and IGFBPs generally inhibit IGF action (25). IGFBP activity is regulated by IGFBP proteases, and proteolysis of IGFBPs is an important mechanism in the regulation of IGF bioavailability (25, 26). Epidemiologic and biological studies suggest IGFBP-3 as an anticancer molecule (27, 28). It has recently been reported that proteolysis of IGFBP-3 by matrix metalloproteinase (MMP)-7 plays an important role in regulating IGF bioavailability (29). Anchorage of MMP-7 to the cell surface may thus provide a mechanism to coordinate IGFBP-3 proteolysis with increased IGF availability in close proximity to the IGF-IR (29–31). We and others reported that MMP-7 plays important roles not only in tumor invasion and metastasis but also in the development and progression of colorectal adenoma tissues (32–34). The absence of MMP-7 reportedly resulted in a reduction in mean tumor multiplicity in Min/+ mice of ~60% and a significant decrease in the average tumor diameter (35). Moreover, it has been reported that local IGF-II supply is a modifier of intestinal adenoma growth in the Min mouse (36).

Thus, it seems important to clarify the relationship between IGFs/IGF-IR axis, COX-2, and MMP-7 in human early colorectal carcinogenesis. We investigated the expression of IGF-II, IGF-IR, IGF-I, COX-2, and MMP-7 in 90 human early colorectal tumor tissues by using the semiquantitative reverse transcriptase-PCR. Loss of imprinting of the IGF-II gene was analyzed by exon-connection reverse transcriptase-PCR and allele specific-PCR (37, 38). Ninety-nine adenoma tissues and 60 pT1 cancer tissues were also analyzed for the expression of IGF-II protein by immunohistochemistry. Moreover, paired carcinoma and adenoma tissue samples obtained from a patient with carcinoma in adenoma lesion were analyzed for expression by a cDNA array.

**PATIENTS AND METHODS**

**Patients and Tissue Samples.** Ninety paired specimens of colorectal tumor and nontumor tissues were obtained by polypectomy or surgical treatment. These tumor samples consisted of 63 adenomas and 27 adenocarcinomas with submucosal invasion (pT1 in the tumor-node-metastasis (TNM) classification of the Union International Contre Cancer). Paired specimens of colorectal carcinoma and adenoma were obtained from 7 patients with carcinoma in adenoma lesion. Each tissue specimen was treated as described previously (39). Additionally, formalin-fixed paraffin-embedded tumor specimens of 99 colorectal adenomas and 60 pT1 cancers were obtained from patients. The histopathological features of the specimens were classified according to the TNM classification system. Localizations of the colorectal tumors were divided into proximal colon (cecum, ascending, and transverse colon) and distal colon (descending and sigmoid colon and rectum). Macroscopic types were divided into protruded type (height of tumor ≥3 mm) and flat type (height of tumor <3 mm). The clinicopathological characteristics of colorectal tumors are shown in Table 1. Informed consent was obtained from each subject, and the institutional review committee approved this study.

**Semiquantitative Reverse Transcriptase-PCR.** Semiquantitative reverse transcriptase-PCR was done as described previously (39). Overexpression was judged when target gene expression in the tumor samples was at least three times higher than that in the corresponding normal sample. The primers used were 5′-AAATCAGCAGTCTTCCAACC-3′ and 5′-CTTCTC

**Immunohistochemistry.** Immunohistochemistry with an anti-IGF-II antibody (clone S1F2, mouse monoclonal IgG1, and 10 μg/mL) was done as described previously (39). Cytoplasmic expression of IGF-II was defined as positive when immunoreactivities were observed in >10% of the tumor.

**Allelic Analysis of IGF-II Expression with the Exon-Connection Reverse Transcriptase-PCR.** We used allele specific-PCR to determined IGF-II Apal genotyping. For measuring IGF-II allelic transcription, the exon-connection PCR in the first-round cDNA-PCR followed by allele specific-PCR was used (37, 38). Two primers spanning exons 8 and 9 were used in a first-round PCR. The PCR product corresponding to the size of the RNA transcript was gel purified and analyzed by allele specific-PCR.

**Analysis of cDNA Gene Expression Profile Analyzed by cDNA Array.** The cDNA array analysis with 5 μg of total RNA was done as described previously (43).

**Statistical Analysis.** Expression of each target gene was assessed for associations with clinicopathological characteristics with the following statistical tests: Student’s t test for age and size, and the χ² two-tailed test or Fisher’s exact test for the remaining parameters.

**RESULTS**

IGFs and IGF-IR mRNA Expression in Colorectal Tumors. To perform semiquantitative reverse transcriptase-PCR analysis, the ranges of linear amplification for each target gene and for the control GAPDH gene were examined. The optimal number of PCR cycles and optimal mixing ratios of primers were determined. The expression of IGF-II, IGF-IR, and IGF-I mRNA in 90 colorectal tumor tissues was examined. Fig. 1 shows representative results. IGF-II mRNA expression was detected in 34 (37.8%) of the 90 colorectal tumor tissues but was
undetectable or only faintly detected in adjacent nontumor tissues. The relationships between IGF-II expression and clinicopathological characteristics are shown in Tables 1 and 2. IGF-II mRNA expression was significantly higher in pT1 cancer (70.4%) than in adenoma (23.8%; P < 0.0001). The expression was correlated significantly with size (P < 0.0162) and age (P = 0.0199). There was no correlation of IGF-II expression with gender, location, or macroscopic type. When only adenoma tissues were considered, IGF-II mRNA expression was significantly higher in flat type (39.3%) than in protruded type (11.4%; P < 0.0099). IGF-IR mRNA expression was detected in 34 (37.8%) of the 90 colorectal tumor tissues but was undetectable

### Table 1 Clinicopathological characteristics and mRNA expression profiles in 90 colorectal tumor tissues

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**NOTE.** Each row is a colorectal adenoma (n = 63) or pT1 cancer (n = 27). Black rectangles indicate each mRNA expression-positive. Abbreviations: M, male; F, female; D, distal; P, proximal; P, protruded; F, flat.
or only faintly detected in adjacent nontumor tissues. The relationships between IGF-IR expression and clinicopathological characteristics are shown in Tables 1 and 2. IGF-IR expression was not correlated significantly with any of the clinicopathological characteristics. IGF-I mRNA expression was detected in 49 (54.4%) of the 90 colorectal tumor tissues and was faintly detected in adjacent nontumor tissues. IGF-I expression was correlated significantly with histopathology ($P = 0.0007$).

There was no correlation of IGF-I expression with age, size, gender, location, or macroscopic type (data not shown).

**Immunohistochemical Expression of IGF-II in Colorectal Tumors.** In positive cases, staining of IGF-II was observed not only at the invasive front but also in the upper part of the muscularis mucosae. IGF-II expression was immunohistochemically positive in 37.7% of the 159 colorectal tumor tissues (Fig. 2). The frequency of IGF-II expression was significantly higher in pT1 cancer (35 of 60, 58.3%) than in adenoma (25 of 99, 25.3%; $P < 0.0001$; Table 2). Immunohistochemical expression of IGF-II was positive in all of the 10 tumors in which IGF-II mRNA expression was detected. In contrast, there was

**Table 2** Immunohistochemical expression of IGF-II and IGF-II and IGF-IR mRNA expressions and clinicopathological characteristics in patients with colorectal tumor

<table>
<thead>
<tr>
<th>Variables/categories</th>
<th>Immunohistochemical expression of IGF-II</th>
<th>IGF-II mRNA expression</th>
<th>IGF-IR mRNA expression</th>
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<tr>
<td></td>
<td>Positive ($N = 60)$</td>
<td>Negative ($N = 99$)</td>
<td>$P$</td>
</tr>
<tr>
<td>Age (years ± SD)</td>
<td>65.8 ± 9.8</td>
<td>64.8 ± 10.2</td>
<td>NS</td>
</tr>
<tr>
<td>Mean size (mm ± SD)</td>
<td>18.8 ± 10.2</td>
<td>20.7 ± 16.1</td>
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<tr>
<td>Gender</td>
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<tr>
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<td>Cancer (pT1)</td>
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<td>25</td>
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</table>

Abbreviation: NS, not significant.
little or no detectable staining in the 10 samples not expressing IGF-II mRNA (data not shown).

Allelic Analysis of IGF-II Expression by the Exon-Connection Reverse Transcriptase-PCR and Allele Specific-PCR. We used allele specific-PCR to determined IGF-II Apal genotype of 90 colorectal tumor tissues. Fifty cases were informative and subjected to exon-connection reverse transcriptase-PCR followed by allele specific-PCR. Loss of imprinting of the IGF-II gene was observed in 15 (44.1%) of the 34 colorectal tumors in which IGF-II was overexpressed. The frequency of loss of imprinting was significantly higher in carcinomas (12 of 19, 63.2%) than in adenomas (3 of 15, 20.0%; \( P = 0.0119 \)). In contrast, normal mucosae and 16 tumor samples without IGF-II overexpression did not show loss of imprinting (Fig. 3; data not shown).

COX-2 and MMP-7 mRNA Expression in Colorectal Tumors. COX-2 and MMP-7 mRNA expression was detected in 38 (42.2%) and 70 (77.8%) of the 90 colorectal tumor tissues but was undetectable or only faintly detected in adjacent non-tumor tissues (Fig. 1). The results of COX-2 and MMP-7 expression in 90 samples are shown in Table 1. COX-2 expression was correlated significantly with size (\( P = 0.0032 \)), gender (\( P = 0.0042 \)), and histopathology (\( P = 0.0004 \); data not shown). MMP-7 expression was correlated significantly with age (\( P = 0.0253 \)), size (\( P = 0.0027 \)), location (\( P = 0.0394 \)), and histopathology (\( P = 0.0057 \); data not shown).

Relationships of Expression of IGFs, IGF-IR, COX-2, and MMP-7. The results of IGF-II, IGF-I, IGF-IR, COX-2, and MMP-7 expression in 90 samples are shown in Table 1. IGF-II expression was correlated positively with IGF-IR (\( P < 0.0001 \)), IGF-I (\( P < 0.0001 \)), COX-2 (\( P = 0.0001 \)), and MMP-7 (\( P = 0.0006 \); data not shown). In addition, IGF-I expression was correlated positively with IGF-IR (\( P < 0.0001 \)), COX-2 (\( P < 0.0001 \)), and MMP-7 (\( P = 0.0027 \); data not shown). IGF-IR expression was correlated positively with COX-2 (\( P = 0.0001 \)) and MMP-7 (\( P = 0.0037 \); data not shown). Finally, COX-2 expression was correlated positively with MMP-7 (\( P = 0.0009 \); data not shown). When only adenoma tissues were considered, these correlations were still significant (data not shown).

The cDNA Array Analysis. In a patient (No. 6 in cancer group) with carcinoma in adenoma lesion, we searched an expression database for genes that were at least 3-fold up- or down-regulated in the carcinoma lesion relative to the adenoma lesion. Two genes and 12 genes were identified as up-regulated and down-regulated genes in carcinoma lesion, respectively (Table 3). Although the gene expression patterns were similar, IGF-II expression level in the carcinoma lesion was >40 times
higher than that in the adenoma lesion. Among the 550 cancer-related genes, IGF-II was the most differentially expressed gene between carcinoma and adenoma lesions. Semiquantitative reverse transcriptase-PCR analysis gave results consistent with those obtained from cDNA array analysis (data not shown). To confirm the results, the expression of IGF-II was additionally analyzed by an immunohistochemical method. Strong expression of IGF-II was seen within the cytoplasm of carcinoma cells compared with that in adenoma cells (data not shown). Moreover, loss of imprinting of the IGF-II gene was observed in the carcinoma lesion but not in the adenoma lesion (data not shown). Considering these results, 6 more individuals with carcinoma in adenoma were then analyzed for mRNA and immunohistochemical expression of IGF-II and loss of imprinting of the IGF-II gene. IGF-II mRNA expression level in the carcinoma lesion was >10 times higher than that in the adenoma lesion in 5 of the 6 patients (data not shown). Thus, when analyzed in total 7 patients including the first patient (No. 6), increased IGF-II mRNA expression in the carcinoma lesion in 6 of 7 patients was statistically significant ($P = 0.0047$). Immunohistochemical expression of IGF-II was positive in all of the 5 carcinomas in which IGF-II mRNA overexpression was detected. In contrast, there was no detectable staining in a carcinoma sample not overexpressing IGF-II mRNA (data not shown). Four cases were informative and subjected to exon connection reverse transcriptase-PCR followed by allele specific-PCR. Loss of imprinting was detected in carcinoma lesion of the 3 patients in which IGF-II was overexpressed but not in a carcinoma sample not expressing IGF-II or adenoma lesions.

**DISCUSSION**

The issue that we addressed in this study was the relationships between the expression of IGFs, IGF-IR, COX-2, and MMP-7 in the early stage of colorectal carcinogenesis. The reason why we chose pT1 cancer is that pT1 cancer represents the early stage of colorectal cancer.

The mRNA and immunohistochemical expression of IGF-II was correlated with each other and observed more frequently in pT1 colorectal cancer than in adenoma, suggesting that IGF-II plays an important role in adenoma-carcinoma progression. When only adenomas were considered, IGF-II mRNA expression was significantly higher in flat type than in protruded type adenomas. This result is interesting because flat type colorectal tumors tend to reach deeper layers earlier and show higher rates of lymphatic invasion and lymph node metastasis than protruded type tumors.

All of the biopsy samples were obtained from the surface of the tumor in this study. Therefore, IGF-II mRNA expression is derived from the tumor cell in the lamina propria mucosae. In addition, by the immunohistochemical method, staining of IGF-II was observed not only at the invasive front but also in the upper part of the muscularis mucosae. Accordingly, it is thought that a tumor in which IGF-II is overexpressed already has malignant potential before it invades the submucosa.

Loss of imprinting is one of the most important mechanisms underlying overexpression of IGF-II in cancer. Indeed, loss of imprinting of the IGF-II gene has been reported in 33% (4 of 12) to 38% (5 of 13) of advanced colorectal cancers (6, 44). In the former analysis, reverse transcriptase-PCR and immunohistochemistry revealed that IGF-II was overexpressed in all of the loss of imprinting-positive cancer tissues compared with its expression levels in noncancerous tissues (6). In this study, among the tumors with IGF-II overexpression, loss of imprinting was detected in 20.0% of the 15 adenomas and in 63.2% of the 19 colorectal cancers with IGF-II overexpression. Thus, loss of imprinting is an important mechanism underlying the overexpression of IGF-II in these early colorectal tumors. However, overexpression of IGF-II can not be explained by loss of imprinting alone. Overexpression of IGF-II can potentially be accomplished by multiple mechanisms, including loss of imprinting, loss of heterozygosity with paternal duplication, excessive transcriptional activation, loss of transcriptional suppression, and alteration in IGFBPs (45). Additional analysis is required to clarify this issue.

Comparison of the gene expression profiles of early invasive cancer and adenoma tissues within a carcinoma in adenoma lesion showed that cancer and adenoma tissues generally exhibited similar gene expression profiles except for several up-

### Table 3 Genes that were at least 3-fold up- or down-regulated in the carcinoma lesion relative to the adenoma lesion in a patient with carcinoma in adenoma lesion (No. 6 in cancer group in Table 1)

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Function</th>
<th>Carcinoma</th>
<th>Adenoma</th>
<th>Carcinoma/Adenoma</th>
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<td>Proapoptotic</td>
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<td>Apoptosis regulator</td>
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<td>17.35</td>
<td>0.42</td>
<td>41.31</td>
</tr>
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</table>

Abbreviations: ARC, apoptosis repressor with a CARD domain; FNK, fibroblast growth factor-inducible kinase; HSP, heat shock protein; Rho GDI, Rho GDP-dissociation inhibitor.
down-regulated genes in cancer tissues, suggesting that cancer developed through a stage of adenoma (46). It is of interest that the IGF-II expression level in the carcinoma lesion was >40 times higher than that in the adenoma lesion. Among the 550 cancer-related genes, IGF-II was the most differentially expressed gene between carcinoma and adenoma lesions. Immunohistochemical IGF-II expression and loss of imprinting of the IGF-II gene were observed in the carcinoma lesion but not in the adenoma lesion. Importantly, similar results were observed in 5 of the 6 patients with carcinoma in adenoma lesion, reaching statistical significance. These results additionally support the notion that overexpression of IGF-II, at least in part, because of loss of imprinting plays an important role in the progression of adenoma to carcinoma. Thus, our results extend roles of IGF-II in the late stage to early stage of colorectal carcinogenesis. Moreover, cDNA array analysis of colorectal cancer and adenoma tissues obtained from a carcinoma in adenoma lesion seems to be useful to clarify relevant alterations of gene expression associated with colon adenoma-carcinoma progression.

Overexpression of IGF-I mRNA expression was observed in 54.4% of the 90 tumor samples, and it was correlated with histopathology. Michell et al. (4) previously reported that IGF-I mRNA level was not differentially expressed between 10 colorectal cancer and normal tissues. Freier et al. (5) reported no evidence of IGF-I mRNA in either 10 cancer or 19 normal tissues. In contrast, Tricoli et al. (2) reported a 3- to 5-fold increase in IGF-I mRNA level in 20% of colorectal cancer tissues. Bustin et al. (47) also reported that IGF-I mRNA levels were higher in cancer than in normal tissues in 31% of the 22 samples. The discrepancy may be because of the few samples analyzed in previous studies and/or differences in the methods of measurement.

With respect to the relationships of IGF-II with IGF-IR, IGF-I, COX-2, and MMP-7 expression, a significant association was found among the expressions of these molecules. We observed a progressive increase in the synchronous expression of IGF-I, IGF-II, IGF-IR, COX-2, and MMP-7 during the transition from normal to adenomatous to carcinomatous colonic mucosa. The synchronous expression of IGF-I, IGF-II, IGF-IR, and MMP-7 in a subset of adenomas and in the majority of early invasive colorectal cancers is consistent with an auto-paracrine loop of tumor cell autostimulation. Colon tumor cells may grow by an autocrine loop mechanism in which the tumor cells overproduce IGFs, which in turn bind to and activate the IGF-IR, on the same tumor cells. MMP-7 may facilitate IGF bioavailability through its IGFBP-3 protease activity (29). COX-2, through PGE3, activates the PI3k/Akt pathway that stimulates IGF-IR expression (20). Up-regulation of COX-2 expression by IGF-II mediated through activation of IGF-IR has also been shown in colon cancer cells (48). It is thought that the PI3k/Akt pathway is activated in tumors in which COX-2 and IGF-II are overexpressed. Thus, the interplay of IGFs, IGF-IR, COX-2, and MMP-7 plays key roles in the early stage of colorectal carcinogenesis. Nevertheless, our results also suggest that this interplay may not be essential for development of a subset of colorectal cancers.

Identification of IGFs, IGF-IR, COX-2, and/or MMP-7–positive colorectal tumors might be beneficial for predictive purposes, as new molecular therapeutic approaches are aimed at interference with the IGF system and related pathways, including COX-2 and MMP-7. Our results additionally support the notion that targeting of COX-2 and IGF-IR by NSAIDs is a potentially promising strategy for chemoprevention. Nevertheless, alterations of IGFs levels or IGF-IR signal transduction reportedly influence the antiproliferative actions of COX-2 inhibitors and attenuate their activity (49). It would be reasonable to presume that agents which interrupt multiple, rather than single, signal transduction pathways will become part of future therapeutic procedure. Thus, the combination of COX-2 inhibitors and disruption of the IGF pathway, through the blockage of receptors or inhibition of secondary targets, would be promising therapeutic strategies (50).

REFERENCES


Interplay of Insulin-Like Growth Factor-II, Insulin-Like Growth Factor-I, Insulin-Like Growth Factor-I Receptor, COX-2, and Matrix Metalloproteinase-7, Play Key Roles in the Early Stage of Colorectal Carcinogenesis
